

EXHIBIT E

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: :
Pieczenik : Group Art Unit: 1805
Serial No. 07/662,764 : Examiner: Lebovitz
Filed: February 28, 1991 :
For: :

DECLARATION OF GEORGE PIECZENIK UNDER 37 C.F.R.1.131

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I, GEORGE PIECZENIK, hereby declare:

1. That I am the inventor of the subject matter of the above-identified patent application and that I am the author of the draft manuscript, a photocopy of which is presented herewith as Exhibit B;
2. That I transmitted the draft manuscript marked Exhibit B to Roy Alan Durham in Los Angeles, California, on a date prior to August 1990;
3. That I instructed Roy Alan Durham, of Bingo BioTech, with whom I have a business association, to send the manuscript marked

Exhibit B by facsimile to Lorance L. Greenlee of Greenlee and Associates on a date before August of 1990;

4. That I knew at the time the manuscript was prepared that it was possible to chemically synthesize a population of oligonucleotides of substantially random sequence and that a library of random coding oligonucleotides could be inserted into a vector such as bacteriophage f1 using recombinant DNA technology, preferably inserted within a structural gene and inserted in the correct reading frame, with the result that such recombinant bacteriophage would express a library of epitopes consisting of substantially random amino acid sequences.

5. That I presented the information and concepts as set forth in the manuscript marked Exhibit B, together with additional information related to the insertion of an endoplasmin epitope coding sequence into the protein III gene of bacteriophage f1 and the reaction of that recombinant protein III with antibody specific for endoplasmin protein, as described more fully below, at meetings with potential corporate research sponsors in the United States on dates after the filing of USSN 770,390, filed August 25, 1985, and prior to August 1990. Participants at these meetings were bound by confidentiality. I further declare that Roy Durham was present at these meetings and that I communicated this information to him at these meetings held in the United States prior to August 1990.

I presented the results of experiments which showed the feasibility of creating an epitope library on the surface of a bacteriophage, where the epitopic sequences each comprised five amino acids. It had been previously determined by others that a primary epitope for the protein endoplasmin within the N-terminal 18 amino acids of the protein corresponded to amino acids 2-6 (Asp-Glu-Val-Asp-Val). In my experiments, an oligonucleotide comprising the nucleotide sequence coding for this epitope was chemically synthesized and inserted in gene III of bacteriophage f1. The recombinant bacteriophage was isolated and the nucleotide sequence encoding the epitope was determined to be GATGAAGTTGATGTTGAT. Phage containing two copies of this sequence in tandem were also identified. A recombinant bacteriophage containing a nucleotide sequence encoding amino acids 1-5 (termed the 1-5 epitope) of endoplasmin (Asp-Asp-Glu-Val-Asp) was also made; this served as a control for specificity.

The phage containing the sequence encoding the 1-5 and the 2-6 epitopes were grown, isolated and purified by polyethylene glycol precipitation and ultracentrifugation. The recombinant phages were solubilized in Ponceau S (0.2% w/v in 7% acetic acid) and run on a SDS-polyacrylamide gel (as described in Laemmli (1970) *Nature* 227:683) in lanes alternating with the pure endoplasmin protein. It was the endoplasmin protein which was used to elicit the endoplasmin-specific polyclonal antibody. The polyclonal antibody which was used in the immunoblotting of the aforementioned

electropherogram was purified by peptide (epitope 2-6) affinity purification; thus, this polyclonal antibody preparation was believed capable of binding all possible conformational epitopes of the peptide. The proteins resolved by SDS-polyacrylamide gel electrophoresis described above were transferred to a nitrocellulose sheet by electrophoresis. The nitrocellulose sheet was then washed with a Marvel-Cadbury's milk powder solution in PBS. This treatment gave a clean background.

The antibody bound to the nitrocellulose sheet was visualized as described in the Specification at page 47.

Figure 1 attached hereto, is a photocopy of a photograph of the autoradiogram of the resulting Western blot. Lanes 1, 3, 5 and 7 were loaded with a preparation of endoplasmin protein, which appears to have partially proteolyzed. Lane 2 was loaded with the recombinant f1 phage containing the nucleotide sequence encoding the 2-6 epitope inserted in gene III. Lane 4 and lane 6 were loaded with the recombinant f1 with the oligonucleotide insert encoding the 1-5 epitope and the complement of the sequence encoding the 2-6 epitope, respectively.

Lanes 1, 3, 5 and 7 containing authentic endoplasmin, bound the radioactive label present in the Protein A, which binds to immune complexes. The binding of the labelled Protein A indicates that the endoplasmin-specific antibody bound. The arrow at the

left of the figure points to a labelled band of roughly the predicted size for the gene III product in Lane 2. The antibody specific for the endoplasmin 2-6 epitope was raised in a rabbit. No antibody is bound at a corresponding location in lanes 4 and 6, showing that the antibody does not bind to the gene III protein nonspecifically. I believe that this was the first demonstration that an epitope could be recognized by its cognate antibody when inserted within a heterologous protein, as well as an individual peptide or in its native state at the N-terminus of the protein in which it occurs in nature.

I also showed at the aforementioned meetings an exhibit, attached hereto as Figure 2 (a photocopy of a photograph of the autoradiogram), which demonstrates that a cognate peptide could compete for the binding of a specific antibody. Figure 3, submitted herewith, is a schematic representation of the autoradiogram shown as Figure 2. Aliquots each containing 1.7×10^{13} recombinant f1 bacteriophage into which the oligonucleotide encoding the 2-6 epitope (EP phage) was inserted was spotted onto nitrocellulose and Hybond (nylon) membranes in wells (Rows 1 and 2, respectively). Aliquots each containing 1.7×10^{13} of the recombinant f1 bacteriophage into which the complement of the endoplasmin 2-6 epitope (EP compl. phage) was inserted were spotted onto nitrocellulose and Hybond membranes (Rows 3 and 4, respectively). 200 μ l of antiserum, which was affinity-purified using the endoplasmin 2-6 amino acid epitopic sequence immobilized

on a column support, was added to each spot and incubated 40 min at 25°C. To Columns 4 and 5, 10 µg and 100 µg, respectively of the peptide corresponding to the endoplasmin 2-6 amino acid sequence were added and incubated for 30 min at 25°C. 200 µl liquid was then removed from each spot. Then 200 µl of I¹²⁵-labelled Protein A was added to each spot and incubated 10 min at 25°C. Then each well was washed with 500 µl TMT (TBS/2% Marvel Cadbury's milk/0.5% Tween 20). An autoradiogram was then prepared from the reacted grid.

The results on the Hybond gave approximately equivalent amounts of radioactivity bound for the recombinant phage comprising the sequence encoding the endoplasmin epitope and for the recombinant phage comprising the complement of the endoplasmin coding sequence. Furthermore, the addition of the peptides did not appear to affect the amount of radioactive Protein A bound. On the nitrocellulose there was no significant amount of label bound to where the EP compl. recombinant phage were bound as compared with where the EP recombinant phage were bound. This suggests that nitrocellulose allows for the detection of a specific endoplasmin 2-6 antibody binding event. In addition, at the positions where the endoplasmin epitopic peptide was added after antibody binding, there appears to have been a diminution of radioactive Protein A bound, suggesting that the peptide competed with the recombinant EP phage for antibody binding. This conclusion is supported by the observation that in Row 1, Column 5, there was less radioactivity

bound than in Row 1, Column 4; it appears that the 10-fold greater amount of peptide in Column 5 resulted in greater displacement of the antibody.

The experimental results (as shown in Figure 2, which is a photocopy of a photograph of the resulting autoradiogram) and more clearly modelled in the schematic representation in Figure 3, show that particular peptide epitopes can be detected when expressed within a vector protein, as exemplified by pIII of f1 and the endoplasmin epitope. The autoradiogram looked substantially like the schematic with respect to relative spot intensities.

I concluded at the time the experiment was completed that the results obtained for the model system of the endoplasmin epitope demonstrated the operability of the invention as disclosed and claimed in USSN 770,390, filed August 25, 1985, the application from which priority in the instant application is ultimately claimed.

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and

that willful false statements may jeopardize the validity of this application or any patent issuing therefrom.

Date: _____

GEORGE PIECZENIK

Exhibit E, Fig. 1
(re: USSN 07/662,764)

1 2 3 4 5 6 7

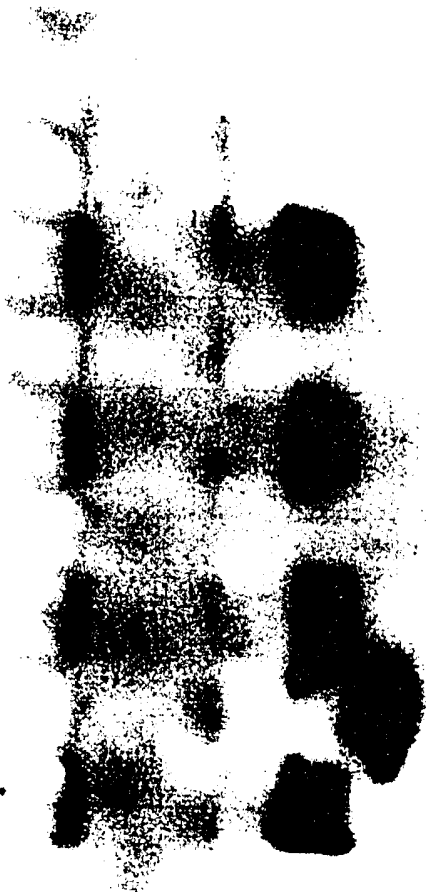
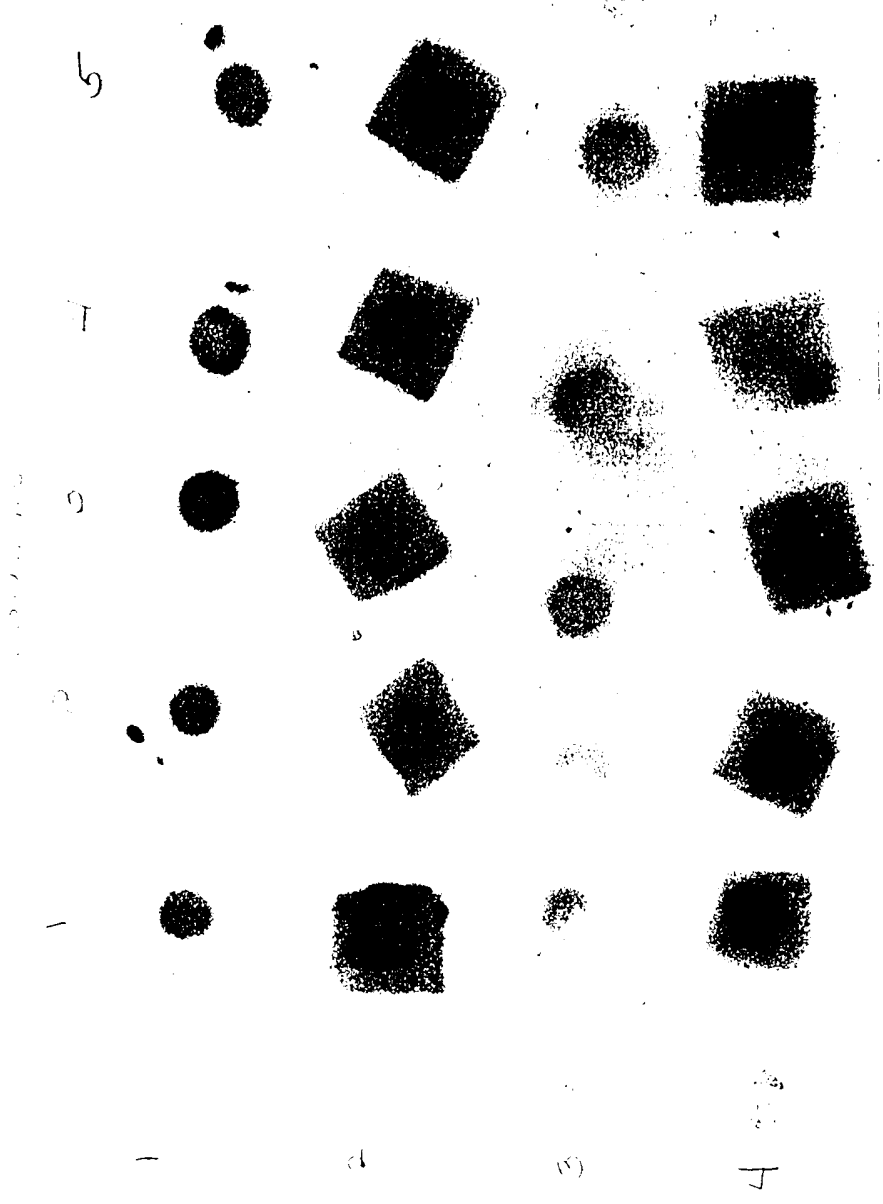
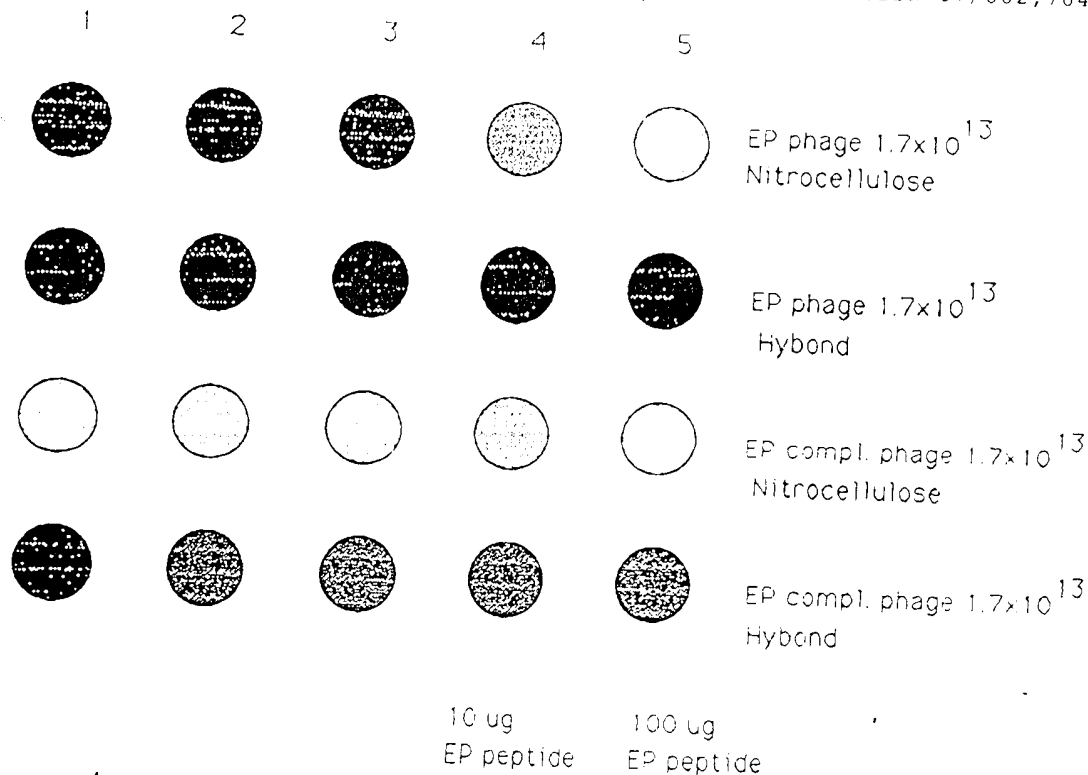


Exhibit E, Fig. 2
(re: USSN 07/662,764)





- 1) 200 ul of anti-sera purified by EP peptide added to each well, for 40 min at 25C. 200 ul then removed.
- 2) 200 ul of protein A 1-125 (1/250 dil). 10 min at 25C.
- 3) Wash 3x with 500 ul TMT (TBS/2% Marvel/5%Tween 20)
- 4) Put film on for 5 days.

Results.

Hybond does not work

EP peptide competes off Ab binding EP sequence on phage. More competition at higher concentration of peptide.

AB does not bind phage coding for complement of EP sequence (EP compl. phage). Therefore labeled protein A does not bind AB.